

The Effectiveness of Postnatal Handling for Maternal Behaviour
Enhancement in Methadone-exposed Offspring

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Abstract

Previous research into the effects of methadone exposure have primarily focused on the prenatal effects, specifically neurological changes and short term physical development. However, it is well known that early mother-child interactions are a vital element to the behavioural and cognitive development of the child. Similarly, it is well known that maternal substance abuse can alter the quality of this interaction. While there have been some studies into the short term postnatal effects on behavioural development, few have looked into the effects methadone might have on maternal behaviour. The present study, therefore, aimed to assess the extent to which the detrimental effects that methadone may have on maternal behavior could be moderated using the postnatal handling paradigm in the long-term by examining the behavioural and cognitive outcomes of the offspring. Methadone was provided in the drinking water of drug-treated dams during lactation (3.0mg/kg/day). The conditions were methadone-exposed/handled (N = 22), methadone-exposed/non-handled (N = 14), control/handled (N = 14), control/non-handled (N = 14). Handled offspring were separated from their mothers for 3 minutes per day for 28 days. Behavioural and cognitive measures (Open field, Zero maze and Y-maze tests) were then taken at postnatal days (PND) 30, 60 and 120. Results showed that methadone-exposed/handled offspring generally presented lower levels of anxious behavior and spatial memory deficits than those in the non-handled group. While the anxiety effects of handling seemed to last into adulthood, handling effects on spatial memory did not. The findings in this study support the need for more research into understanding the risk factors associated with the effect of methadone on maternal behaviour and the possibility to reverse these effects by taking measures to improve maternal-infant interactions

1.0 General Introduction

The current study aimed to assess the extent to which postnatal handling could moderate the detrimental effects that methadone might have on maternal behaviour in the long-term by examining the behavioural and cognitive outcomes of the offspring. This introduction will firstly discuss the prevalence of opioid use, particularly in women of childbearing age, as well as its effects on exposed children. This will then be followed by a discussion about the methadone maintenance therapy program as well as current research on the effects of methadone, particularly in terms of pre- and postnatal effects both in human and animal samples. Current research on the influence of maternal behaviour on childhood development will then be discussed with specific emphasis on maternal substance use. Following this, the postnatal handling paradigm will be introduced. Finally, after discussion of the research as it stands, the focus of the present study will be addressed.

1.1 Opioids

Opioid drugs such as morphine, heroin, codeine and oxycodone are derived from opium, a powdered exudate of the poppy plant, *papaver somniferum*. Opioids are powerful analgesics and as such are typically used to relieve pain. In addition to analgesic properties, opioids are also well known to produce euphoria, which is the primary reason individuals engage in recreational use (Fodor, Tímár, & Zelena, 2014).

Opioids are effective by acting on the opioid receptors in our body. Opioids inhibit adenylylase activity and the cyclic adenosine monophosphate activity (Chhabra & Bull, 2008). Continual use of opioids, leads to the development of tolerance as result of compensatory increases in cyclic adenosine monophosphate activity and adenylylase levels due to influx of

intracellular calcium ions and increased NMDA activity. Consequently, the user would require increasing amounts of the substance to achieve the desired euphoric effects, resulting in dependence. The abuse of opioid drugs can be potentially dangerous and could bring about a number of negative effects on the user, such as, engaging in criminal activity in order to fund the habit as well as a heightened risk of human immunodeficiency virus (HIV) through needle sharing (Devi, Azriani, Wan, Ariff, & Hashimah, 2012).

In 2016, the United Nations reported that the use of opioids, particularly heroin, in the United States has increased from 20.7% in 2002-2004 to 45.2% in 2011-2013 (UNODC, 2016). In New Zealand, approximately 10,000 adults have an opioid dependency, of which approximately 50% are enrolled in opioid substitution treatment (OST) services (Adamson et al., 2012; Deering, Sellman, & Adamson, 2014). Women were reported to be more likely to engage in the recreational use of prescription opioids and tranquilizers than men. Over the past decade the prevalence of heroin use among women in the United States has doubled from 0.08% in 2002-2004 to 0.16% in 2011-2013. This increase was significantly higher in young men and women aged between 18 and 25 years. For females of childbearing age, the exposure to opioids may negatively impact the offspring. This is because opioids receptors are present in several areas of the brain and multiple mechanisms can be affected by opioid exposure. Morphine, among other opioids, can cross the placenta, the blood-brain barrier and exude into the breast milk (Fodor et al., 2014).

Maternal opioid use has been reported to result in harmful medical and social consequences for both mother and infant. Both opioid intoxication and acute withdrawal can cause harm such as an increased risk of spontaneous abortion, stillbirth, prematurity, and neonates born with low birth weight, undesired neural effects, or birth defects. Furthermore,

abrupt withdrawal from opioids can lead to premature labour, foetal distress and foetal withdrawal symptoms (Tran, Griffin, Stone, Vest, & Todd, 2017)

Animal studies have found that the offspring of dams exposed to morphine during pregnancy displayed increased anxiety-like behaviour possibly as a result of altered prolactin regulation (E. M. Byrnes, 2005; Fodor et al., 2014). In humans the effects of prenatal exposure include stillbirths, sudden infant death syndrome whereby the principal causes of death are prematurity and growth retardation (Bashore et al., 1981). Other effects include a reduced head circumference, lower birth weight and smaller body lengths (Daly, Hughes, & Woodward, 2012; Fodor et al., 2014). In addition, studies have shown that foetal exposure to opiates may adversely affect the migration and survival of neurons which could lead to developmental complications during this period (Walhovd et al., 2009). Other studies have shown long term consequences of prenatal morphine exposure namely, changes in motor development and signs of impaired spatial memory.

1.2 Methadone

Methadone (6-dimethylamino-4, 4-diphenyl-3-heptone) is a synthetic opioid, which mechanism of action is primarily mediated by the activation of the μ -type opioid receptors. It is usually administered orally as a racemic mixture of two enantiomers: R-methadone and S-methadone. Studies have clearly demonstrated that methadone is an effective treatment for opioid dependency thereby reducing illicit drug use, crime, mortality and unemployment (Eap, Buclin, & Baumann, 2002)

1.2.1 Pharmacokinetics

Methadone is primarily a μ -receptor agonist and may mimic the endogenous opioids and endorphins which affect the release of other neurotransmitters such as acetylcholine, norepinephrine and dopamine. Methadone differs from morphine by an additional weak antagonist activity at the N-methyl-D-aspartate (NMDA) receptor. It contributes to the prevention of withdrawal effects and reduction of morphine cravings as a result of this NMDA antagonism which attenuates and reverses the development of tolerance to morphine without altering its analgesic properties (Eap et al., 2002). This accounts for the analgesic and antinociceptive outcomes as well as other side effects such as respiratory depression, sedation, and decrease in bowel motility to name a few (Anderson & Kearney, 2000).

In addition to opioid receptors, methadone binds to plasma proteins to a high degree, predominantly the α 1- acid glycoprotein (AAG) (Garrido et al, 2000). AAG exhibits variations in its plasma levels depending on physiological or pathological conditions. It is generally recognised that during stressful conditions, AAG levels show a significant increase. Such an increase was found to be the main factor responsible for lower free fractions in plasma for methadone in opioid addicts compared with healthy volunteers (Garrido et al, 2000). Methadone is a highly lipophilic molecule allowing it to be widely distributed in the body tissues (Layson-Wolf, Goode & Small, 2002). Methadone maintenance is known to be an effective treatment for opioid addiction as it has a long half-life and can be detected in the blood within 15-45 minutes after oral administration (Eap et al, 2002; Garrido & Troconiz, 1999; Farid, Dunlop, Tait, & Hulse, 2008). Peak plasma concentrations would be reached 2.5-4 hours after ingestion.

1.2.2 Methadone Maintenance Therapy (MMT)

The aim of MMT is to substitute methadone for heroin, which is associated with high risk of morbidity and mortality as well as to improve the quality of life of those who are opioid

dependent by reducing relapses as well as improving their physical and mental condition (Anderson & Kearney, 2000; Devi et al., 2012). Patients are initially given a low dose of methadone at the start of treatment which usually ranges between 10-30mg. The dose can be increased after three days if the patient begins to exhibit signs of withdrawal up to a maximum dose of 20 mg a week. Patients are required to present themselves at a clinic or pharmacy to receive their daily dose, and because MMT is a long-term treatment, patients receiving methadone will have to do so for several months to years (WHO, 2009). Methadone dosage is strictly managed daily by a registered health professional in order to minimise diversion, which is the act of selling or giving away of methadone to others.

Should a patient wish to discontinue methadone treatment, it is recommended that they are weaned off the drug over a period of a few weeks with the help of a registered health professional. However, cessation of treatment during pregnancy is not recommended as this could increase the risk of withdrawal symptoms in the mother and foetus as well as stillbirths (WHO, 2009; Albright et al., 2011). In fact, several studies have suggested that the dosage of methadone for pregnant women should be increased in order to accommodate the physiological changes experienced by a woman during this period which could alter the pharmacokinetics of methadone, particularly its absorption, metabolism, distribution and elimination (Shiu & Ensom, 2012; Albright et al., 2011). In addition to the daily dose of methadone, some MMT programs include psychosocial treatments as well. A recent review found that an integrated approach to treatment, whereby pregnant women are provided with obstetric and addiction care with the help of physicians, nurses and counsellors are highly beneficial for their well-being (Ordean, Kahan, Graves, Abrahams, & Boyajian, 2013).

1.2.3 Treatment Outcomes

The methadone abstinence syndrome is prolonged but less severe than for heroin due to its longer half-life (20-35 hours) and duration of action (Shiu & Ensom, 2012). Methadone maintenance therapy has been found to prevent opioid withdrawal symptoms and block the euphoric effects of heroin, hence, minimising the craving for heroin. As a result, methadone maintenance therapy allows a person to reintegrate as a functional member of society by offering reprieve from the daily life associated with procurement and use of heroin (Devi et al., 2012; Anderson & Kearney, 2000; Mattick, Breen, Kimber, & Davoli, 2009).

In 2009, the World Health Organisation Guidelines recommended methadone and buprenorphine as the first line of defence for opioid maintenance treatment (WHO, 2009). Research has shown that MMT is useful for maintaining retention in treatment, as well as mental and physical well-being of its clients i.e. it reduces the use of illicit drugs, HIV risk behaviours, criminal activities and improve employment outcomes (Garcia-Portilla, Bobes-Bascaran, Bascaran, Saiz & Bobes, 2012; Sun et al., 2015).

1.3 Prenatal Effects of Opioid and Methadone Exposures

The abuse of opioid drugs during pregnancy can have serious consequences for the developing foetus and newly born infant. In general, studies describe the negative impact on interuterine growth, metabolism and neonatal behaviour. Differences in the effects of heroin and methadone on these variables are partly due to differences in the direct effects of the drugs, and partly due to differences in nutrition, antenatal care and general life styles among women addicted to these two agents.

Opioids undergo rapid transplacental passage (less than 60 min), and as a result, maternal and foetal signs of withdrawal begin to show within 48 hours after the last use (Bhuvaneswar,

Chang, Epstein, & Stern, 2008). Because it readily crosses the placenta, maternal heroin use during pregnancy is associated with an increased risk for a number of adverse neonatal outcomes, including low birthweight, antepartum haemorrhage and increased neonatal mortality as a result of prematurity and growth retardation (Farid et al., 2008; Fodor et al., 2014; Bashore, 1981). Animal studies have suggested that foetal opioid exposure can potentially have adverse effects on the survival and migration of neurons which can lead to an inhibition of brain growth and central nervous system development (Walhovd et al., 2009). Similarly, Lu, Liu, Long, & Ma, (2012) suggested that heroin use during pregnancy can cause morphological alterations to pyramidal neurons in the somatosensory cortex which could impair short-term spatial memory in rat offspring.

On the other hand, prenatal methadone exposure is widely known to result in withdrawal effects in the infant commonly known as neonatal abstinence syndrome (NAS). NAS tends to occur in 55-94% of neonates exposed to opioids in utero regardless of sex (Fodor et al., 2014; Jansson et al., 2007). Commonly observed symptoms include irritability, high-pitched crying, tremors, hypertonicity, vomiting, diarrhoea and tachypnea. The onset of signs of withdrawal from heroin often begins within 24 hours of birth, whereas, withdrawal from methadone can be delayed up to 72 hours (Logan, Brown, & Hayes, 2013). During gestation, mothers using illicit opioids or on medication-assisted treatments using opioid agonists such as methadone, expose the foetus to opioid dependence (Tran et al., 2017). Infants who experience symptoms of NAS may be less responsive to social interactions and difficult to comfort during the first few weeks of life. This in turn may compromise the relationship with the primary caregiver, especially with vulnerable caregivers (Konijnenberg, Sarfi, & Melinder, 2016).

Nevertheless, MMT, when combined with good antenatal care and supervision of illicit drugs, produces a considerable improvement in birth-weight and decreased risk of neonatal morbidity, when compared to the outcomes of neonates from non-treated pregnant heroin users (Farid et al., 2008; Hulse, Milne, English, & Holman, 1997).

1.4 Postnatal Methadone Exposure and Breastfeeding

Human milk provides optimal nutrition for a newborn baby, and the act of breastfeeding is associated with improved health for the infant and mother and subsequently, an enhanced early mother-infant attachment (Welle-Strand et al., 2013). Lactation is linked with the suppression of stress-responsive hormone secretion and a short-term suppression of the cortisol response to mental stress. Hence, breastfeeding may serve to reduce the overall stress response and enhance maternal self-esteem by allowing a mother to provide optimal nutrition for her infant. In addition to promoting interaction and bonding between a mother and her infant, it provides protection from disease pathogenesis in the short term, supports the development of a healthy immune system in the long term, as well as providing protection from the sudden infant death syndrome (Gregory & Walker, 2013; Jansson, Velez, & Harrow, 2004).

Nevertheless, women on the MMT program receive inaccurate and inconsistent advice from healthcare professionals about breastfeeding. It was recently reported that many women on the MMT program are advised to discontinue nursing because of concerns about possible adverse effects on their infants (Welle-Strand et al., 2013). Such advice may not be entirely accurate due to the limited information available about the extent of excretion of a particular drug into human milk. In order to provide accurate advice, health practitioners would have to consider certain factors such as, the amount of the drug excreted into human milk, the extent of

oral absorption by the infant as well as the potential adverse effects on the breastfeeding infant (Sachs, 2013).

Illicit drugs reported to cause adverse effects to infants via breast milk exposure include heroin, alcohol, cannabis and benzodiazepines. Heroin-exposed infants experienced withdrawal symptoms, tremors, restlessness, vomiting and poor feeding (Sachs 2013). Similarly, animal studies have shown that in addition to slower weight gain, male rats experienced impaired habituation to a novel environment and female offspring performed poorly in a passive avoidance retention task after being exposed to morphine postnatally through breast milk (Nasiraei-Moghadam et al., 2013; Timár et al., 2010).

A recent study conducted by Vestal-Laborde, Eschenroeder, Bigbee, Robinson, & Sato-Bigbee, (2014) found that perinatal exposure to therapeutic doses of methadone can alter early myelination in the developing rat brain. The formation of myelin is a crucial process to support brain maturation and myelin-forming oligodendrocytes have been identified as critical players in the support of neuronal survival and axonal function and integrity. At various maturational stages of oligodendrocytes, neural stem cells express opioid receptors and their modulation has been shown to induce mitogenic and differentiating effects. On this basis, the authors suspected that interference in the endogenous opioid system by opioid drug abuse and maintenance treatments may have an effect on brain myelination. In this study, the rat pups were exposed to methadone during pregnancy and postnatal development. Pups were then sacrificed and their brains examined at postnatal days 11 and 19. Exposed pups showed elevated levels of myelin basic proteins, myelin proteolipid protein and myelin-oligodendrocyte glycoprotein. Such accelerated and increased oligodendrocyte maturation and myelination could potentially disrupt normal connectivity in the developing brain (Vestal-Laborde et al, 2014). This study examined the

effects of a combined exposure to methadone prenatally and postnatally and highlighted the vulnerability of early stages of brain development.

The properties that allow increased passage of drugs into breast milk via passive diffusion include lower molecular weight, being highly lipophilic and minimal protein binding (Holmes, Schmidlin, & Kurzum, 2017). Methadone has these properties, making it able to pass through breast milk with ease. Jansson et al (2007) evaluated the methadone content in breastmilk in the immediate postnatal period. They found that the concentration of methadone in breast milk was small even at peak maternal plasma methadone levels, ranging between 20.6 to 314.2 ng/ml, and was not dependent on maternal dose. This study suggested that individual maternal doses of methadone should not be a factor to discourage women to breastfeed and supports the recommendation that women continue to breastfeed while undergoing the methadone maintenance program. Jansson et al (2007) concluded that the benefits of breastfeeding outweighed any risk of adverse effects from methadone exposure through breast milk. However, it should be noted that this study consisted of a small sample of participants and had inconsistent sampling for the first day of life because of difficulties in collecting colostrum.

These conflicting findings highlight the lack of research in this area and consequently, the need to understand the extent to which methadone exposure through lactation can affect developmental outcomes in newly born infants. Such information is crucial in order to provide best advice lactating mothers on the risks or lack thereof of breastfeeding.

1.5 Influences of Maternal Behaviour on Development

Apart from the risk factors associated with methadone exposure during pregnancy and lactation, past research has suggested that extraneous factors such as maternal behaviour can play

a significant role in the behavioural and cognitive development of a child. During the early stages of postnatal life, maternal care is the main source of environmental stimuli for the offspring as well as a major determinant of behaviour in adulthood (Fenning & Baker, 2015; Stamatakis et al., 2015). It is commonly accepted that the quality and quantity of care received by a child can determine its emotionality, cognitive and social skills well into adulthood.

Several mechanisms have been implicated in the role that negative child-caregiver interactions play on the development of children's social, emotional and cognitive adjustment. Firstly, an infant may not experience the maternal emotional availability and support needed to help in learning to develop the necessary self-regulation skills to regulate and control their own emotions and behaviours (World Health Organization, 2004). This self-regulatory behaviour can take away time from exploring, learning, and engaging in developmentally appropriate and meaningful social interactions with others (Tronick et al., 2005). Secondly, caregiver-child interactions have the potential to shape mental representations of the self, as well as other people, which can then influence assistance-seeking behaviour among others. Studies have shown that these interactions are important for learning and social engagement. For example, children may stop seeking the help of others if caregiver-child interactional failures are experienced repeatedly (Konijnbergen et al., 2016). Moreover, studies have found that NAS symptoms may result in the infant being less responsive to social interactions which may subsequently undermine the relationship with the primary caregiver, as these infants are irritable and difficult to soothe, and therefore parent (Dawe, Harnett, Rendalls, & Staiger, 2003; Maguire et al., 2016).

Psychosocial adversity can further exacerbate the risk for negative outcomes in children of mothers with substance use disorder and these include psychiatric disorders such as depression, stressful family environments, marital discord and caretaker instability (Keller,

Catalano, Haggerty, & Fleming, 2002; Hussong et al., 2009; World Health Organization, 2004).

These factors illustrate that maternal substance abuse could serve as a marker for a range of negative risk factors that could increase a child's risk for poor adjustment (Solis et al., 2012).

However, it is important to note that although some of the risks faced by children of substance-abusing mothers may be uniquely associated with their mother's substance abuse, some of the risks are likely not (Solis et al., 2012). The vast number of confounding factors adds to the difficulty of attributing exposure to a particular drug as a contributing element to developmental difficulties in childhood.

Central to this thesis is the construct of maternal responsiveness that can be conceptualised as multidimensional as it includes contingent reactions, emotional support, encouragement of joint attention and provision of developmentally appropriate structuring and language input (Fenning & Baker, 2012). Maternal responsiveness has been shown to benefit children's cognitive development including children with developmental risks. However, studies have found that the natural joy and reward associated with parenting can be altered as a result of substance abuse (Maguire et al., 2016). Additionally, maternal substance use represents a higher association with parenting ambivalence, decreased sensitivity and responsiveness to the child, increased use of harsh disciplinary practices and neglect (Calhoun, Conner, Miller, & Messina, 2015; Maguire et al., 2016).

In light of the above, it is clear that maternal behaviour can heavily influence a child's behavioural and cognitive development. Although, the challenge now is to determine the extent to which these areas of development can be influenced by detrimental maternal behaviour as a result of drug abuse, the extent to which methadone treatment contributes to behavioural and cognitive impairments is yet to be clearly understood.

Moe (2002) conducted a longitudinal study to evaluate the developmental outcomes of a group of children prenatally exposed to opiates under conditions of minimal postnatal social risk. The biological mothers in this study were all heavy substance abusers, with heroin being the preferred drug and all of the children from this group were placed in foster or adoptive homes within the first year of life. They found significant differences between the substance exposed group and the comparison group on the developmental assessments performed at ages 1 and 4.5 years. The findings showed that although the mean cognitive scores were within normal limits at age 4.5 years, a special weakness in the area of visual-motor and perceptual abilities was detected among the substance-exposed children. The study indicated that even if children experience adequate caregiving, the risk factors associated with prenatal substance exposure is still a potential determinant of developmental problems (Moe, 2002).

In contrast, Konijnenberg et al (2016) found that maternal sensitivity was a better predictor of the quality of a mother-infant relationship than whether or not they were prenatally exposed to methadone. In a longitudinal study, these authors explored whether mother-child interaction moderates the effects of prenatal exposure to methadone on cognitive development and whether prenatal exposure increases children's vulnerability to the effects of poor mother-child interaction. At 12 months of age, the mother-child interaction was measured based on the maternal behaviour (sensitivity/ responsiveness, intrusiveness, detachment/disengagement, positive regard for the child/positive affect, animation and stimulation of development). At 4 years of age maternal behaviour was assessed based on behaviours such as supportive presence, respect for child's autonomy, stimulation of cognitive development, quality of assistance, hostility and confidence. The cognitive development of the children was tested for verbal and spatial reasoning, perceptual alertness, working memory, psychomotor skills, visual attention,

and graphomotor speed and accuracy. This study found that overall, the methadone-exposed groups scored significantly lower on measures of cognitive development and mother-child interaction compared to the comparison group. Specifically, it was found that measures of narrative memory and vocabulary appeared to be more influenced by mother-child interaction. This suggested that differences in mother-child interaction may be more related to differences in language-related cognitive skills while performance in higher cognitive functions which require precise control over sensorimotor responses may be more influenced by methadone exposure-related factors. However, other confounding factors such as prenatal exposure to other substances, like nicotine or alcohol, may also influence cognitive development. It was therefore concluded that a cumulative effect of multiple risks, whereby methadone exposure is one of the factors, could influence the outcomes in the child (Konijnenberg et al., 2016). This is supported by a study conducted by Bernstein and Hans (1994) whereby a group of methadone-exposed and non-exposed children were investigated, all of whom were from low socioeconomic (SES) neighbourhoods. The findings revealed that prenatal methadone exposure alone did not have a negative impact on the developmental outcomes of the children as measured by standardised tests. Instead, the authors concluded that poor mother-child interaction was a better predictor of negative outcomes.

A recent study by Daly et al (2012) found that exposure to methadone was significantly correlated with higher levels of anxiety in offspring only when exposed through both gestation and lactation. This study examined the long-term effects of offspring that were exposed to methadone either during lactation, gestation or both on anxiety-related behaviour as well as the incidence of physical abnormalities. It was found that methadone exposure during gestation alone had little effect on subsequent anxiety of the offspring, whereas, lactational exposure alone

produced increased activity levels. When exposed during both gestation and lactation, rat offspring displayed more anxious behaviour. Furthermore, this study found significant sex differences on all behavioural measures. For example, male rats displayed more anxious and fearful behaviour than females. Additionally, it was found that overall, the rats displayed less anxious and fearful behaviour with age. This suggests that the effect of methadone, regardless of the manner of exposure, may not last into adulthood. Daly et al (2012) suggested that the changes in offspring activity may have been a result of the effect of the drug on maternal behaviour instead.

In rats, both olfactory and tactile signals from the mother impact the developing neonatal brain (Reis et al., 2014). The development of the endocrine, emotional and cognitive responses to stress can be regulated by variations in maternal behaviour, in particular, behaviours such as licking and grooming (Champagne, Francis, Mar, & Meaney, 2003). Past studies have found that olfactory signals from the mother rat can have a marked effect on the developing brain, such as facilitating social learning (Sullivan, Wilson, Wong, Correa, & Leon, 1990). This neural response to maternal odours and tactile stimulation suggest that both the presence of the mother and the care provided by her can significantly alter brain function (Curley & Champagne, 2016). Similarly, the effects of postnatal tactile stimulation have also been observed in preterm infants, who had shown resulting enhancement of growth and neurodevelopment (Curley & Champagne, 2016).

Within the first two weeks of life, the rat will undergo rapid growth, brain functional organisation, neural proliferation, migration and differentiation, gliogenesis and myelination. Humans experience this maturation from the 6th month of pregnancy to the 3rd year of life (Antoniazzi et al., 2017). Overall, there is significant support for the theory that tactile

stimulation received by offspring during mother-infant interactions can influence the development of neural and physiological systems.

1.5.1 Postnatal Handling

In light of the above, many studies have utilised the postnatal handling method to examine the effects of maternal behaviour on the behavioural and cognitive developments in rat offspring. Postnatal or neonatal handling is an experimental paradigm whereby rat offspring are briefly separated from their mother and placed in a new environment for a specified amount of time and subsequently returned to her (Rainecki, Lucion, & Weinberg, 2014). This procedure may be repeated from 10 to 21 days and generally from 1 to 15 minutes at a time (timings vary between laboratories, Rainecki et al., 2014). Several studies have confirmed that postnatal handling does increase maternal behaviours such as licking, grooming of offspring, and nest building (Reis et al., 2014; Champagne et al., 2003). Several studies have found that postnatal handling can alter the Hypothalamic-Pituitary-Adrenal (HPA) axis response to stress as well as improve spatial memory in rat offspring. For example, Macrí, Mason, & Würbel (2004) found that postnatal handling (15 minute separation between mother and offspring) induced a more active nursing style and elevated levels of maternal care and that handled offspring displayed less anxious behaviour, which indicated that postnatal handling attenuated HPA axis responses and fearfulness in adulthood. Additionally, postnatal handling has been shown to have a long-lasting impact on learning and memory, in particular, spatial learning and memory. Stamatakis et al (2008) conducted a study to investigate whether the improved ability of postnatally handled rats to cope with stress would enable them to perform better on a spatial task. They found that this was indeed the case. In particular, the advantage of handling was observed for male rats who had experienced acute stress before as opposed to after a training session.

As a result of the effects found in past studies such as those mentioned above, researchers have begun to examine whether the postnatal handling method has the potential to ameliorate the adverse effects of earlier insults. One study found that postnatal handling (3 minute separation) was able to reverse the behavioural deficits induced in both males and females by prenatal stress (Wakshlak & Weinstock, 1990). This study found that the reduction in anxiety observed in the handled rats lasted until adulthood. In contrast, postnatal handling was not able to entirely reverse the spatial learning or anxiety deficits caused by prenatal alcohol exposure (Hannigan, O'Leary-Moore, & Berman, 2007). These varying results for the effects of postnatal handling suggest that the level of harm experienced in the early life of the offspring may affect the extent of the benefits of handling.

1.6 Summary and The Current Study

The availability of heroin is relatively low in New Zealand. Nevertheless, there was an increase in the proportion of frequent injecting drug users who used morphine from 54% in 2008 to 69% in 2015 (Wilkins, Prasad, Parker, Wong & Rychert, 2017). As mentioned previously, these include women of childbearing age whose offspring may be adversely affected as a result of exposure to such drugs. In general, methadone maintenance programs have largely benefited those who are addicted to opioids. However, there are inconsistencies in the literature regarding the effects of methadone on maternal behaviour and their prenatally exposed infant.

The majority of the studies to date have been conducted with small sample sizes and have predominantly assessed short term impacts of prenatal methadone exposure, whereas, few have investigated the impact of postnatal methadone exposure via lactation. The act of breastfeeding has been acknowledged as being a crucial behaviour, not only for the well-being of the infant, but also for the benefits it brings to maternal mental health and maternal-infant bonding.

Therefore, the advice given to mothers by health care professionals about the safety of breastfeeding while undergoing methadone maintenance treatment should be consistent and reliable. In order to enhance the knowledge in this area, it is important to investigate not just the short-term physical effects of the drug, but the long-term cognitive, emotional and behavioural effects as well.

In general, research in this area has its challenges as a result of various confounding factors such as polysubstance use, maternal behaviour, negative environment and maternal mental illness (Solis et al., 2012). Many past studies have methodological limitations which arose from a lack control for these confounding variables and many were reliant upon self-report data.

The current study was designed to provide more information about possible direct effects of methadone exposure through breast milk on long-term cognitive and behavioural development of both male and female rats from birth to adulthood while controlling for as many environmental influences as possible. However, in view of the effects of environmental stimulation and postnatal handling discussed above, the main aim of the current study was to examine the extent to which postnatal handling might moderate the potentially deleterious effects of methadone on maternal behaviour and thus ameliorate any cognitive and/or behavioural deficits in the offspring.

2.0 Method

2.1 Subjects

20 female PVG Black Hooded rats, approximately 100-150 days old, were housed in individual plastic cages with stainless steel tops (width, length, depth = 35 x 55 x 21cm) with one male each for a period of 2 weeks before the male was removed. They were kept on a 12 hour light-dark cycle at an ambient temperature of 22°C and humidity of 50%. 7 females gave birth, 7 had consumed their pups and 6 did not get pregnant. Due to low numbers of offspring, rebreeding was necessary. The 6 females that did not conceive initially were used for rebreeding. Rats that had consumed their pups were excluded from rebreeding. The breeding process for the second batch involved placing one male with three females in each cage for a period of 2 weeks before the male was removed. These females were then placed into individual cages. The second round of breeding resulted in the birth of 4 litters.

Upon the birth of their litters, the mother rats were randomly allocated as evenly as possible into either the treatment group or control group. 6 mothers on methadone and 5 control. The treatment group mothers had their drinking water replaced with water which contained methadone hydrochloride at a dose of 3mg/kg, and the control group mothers received unadulterated drinking water. Three litters from the methadone group and 2 litters from the control group underwent neonatal handling for 28 days beginning at PND 3. At PND 30 all pups were weaned and housed in group cages of 2 to 4 same-sexed rats each.

Overall there were 4 experimental groups. These were as follows: 1) Methadone-Handled; 2) Methadone Non-handled; 3) Control-Handled; control non-handled. As there were limited numbers of litters born to each group, all rat pups were kept in order to maintain

sufficient numbers of subjects. The number of rats in each of the experimental conditions varied slightly as a result of litter numbers in both control and methadone-exposed groups. The overall numbers were 22 in the Methadone Handled group (14 females and 8 males), 14 in the Methadone non-handled group (9 females and 5 males), 14 in the Control Handled group (7 females and 7 males), and 14 in the Control non-handled group (6 females and 8 males).

2.2 Drug Dosage and Administration

Methadone hydrochloride solution (Biodone Extra Forte Oral Liquid, Biomed Ltd) was purchased from CDC Pharmaceutical Ltd, Christchurch. with a concentration of 10mg/ml. The methadone concentrate was mixed with tap water to produce a diluted solution which was stored in the amount of 5L at a time. The solution was administered at a dose of 3.0mg/kg/day to dams as soon as the births of their litters were noticed. Methadone-treated water was administered via drinking bottles which were attached to their individual cages. The rats had free access to these.

2.3 Materials

2.3.1 Weights and cages

The water bottles and the lactating mother rats were weighed. From the weights of the water bottle it was later possible to calculate the volume of fluid drunk and, for methadone-treated rats, the dose of drug consumed. Cages were lined with wooden pellets and shredded paper for the rats to nest for the purpose of alleviating some of the potential stress associated with isolation.

2.3.2 Handling

The handling procedure involved placing rat pups into a small round plastic bowl which was 18 cm in diameter and had a height of 5.7 cm. Once pups reached 2 weeks of age they were placed into a regular sized cage (dimensions as mentioned under “Subjects”) for the handling period.

2.3.3 Zero maze

The wooden, circular zero maze was 105 cm in diameter with a 10 cm wide platform and had four equal sized quadrants. Two of the enclosed quadrants were opposite to each other and had 25 cm aluminium walls on both inner and outer sides which were painted black. Similarly, both open quadrants were opposite to each other and had a white platform with a small 1 cm high clear Perspex lip on each side designed to prevent rats from falling off. The maze was elevated 72 cm above the floor.

2.3.4 Y maze

The wooden Y-maze had two arms. Both the right and left arms were 45cm in length and then stem was 15cm long. Both arms and the stem were 10cm wide and 14 cm high. The right and left arms were separated at an angle of 120 degrees. The top part of the entire maze was covered with a clear Perspex lid connected by a hinge. The aluminium inserts used inside the maze were painted black and white; each occupied the entire width and height of each arm and was 40cm in length. The inserts covered the side walls and floor of the maze.

2.3.5 Open field

The square wooden open field chamber measured 600x600mm and a height of 250mm. The walls and floor of the chamber were painted black with white intersecting lines painted on

the black floors which divided the floor into 16 equal squares, thus creating a 4x4 grid. Each square within the grid was numbered 1 to 16.

2.4 Procedure

After mating, each female rat was then placed into an individual cage, as mentioned above. All rats were supplied with plain tap water throughout their pregnancy. Upon the birth of their litters the dams were randomly assigned to either the Methadone group or the Control group. A day after the birth of their litters, water bottles were replaced with either methadone solution or plain tap water depending on group allocation. All filled water bottles were weighed to measure the start amount and again at the end of each week to measure the end amount for enabling calculation of the individual weekly fluid intake as well as the methadone dose for the 6 methadone-exposed dams. Body weight of the dams was not measured until the second week of lactation to reduce the stress after birth. Body weights were measured weekly. Bottles were refilled and weighed weekly and topped up when necessary. These body and bottle weights were used to calculate individual daily dosage of each mother rat.

2.4.1 Handling

The litters were randomly allocated to Handled and Non-handled groups after birth. Pups in the non-handled group were not handled until the behavioural testing phase except when being transferred into clean cages every fortnight. Handling did not begin until PND 3 to avoid any additional stress on the mother after the birth process. Each mother in the handled group was removed and placed in a separate cage before her pups were removed and placed in a plastic container containing soiled nesting material from their own cage to provide some insulation from the plastic container. Handled pups were left in the container for 3 minutes then returned to their

home cage before the mother was reunited with them (Raineke et al., 2014). This process occurred daily for 28 days after which the mothers were removed from their litters and rats from each litter was caged in same-sexed groups, as described in “Subjects”.

2.4.2 Behavioural Testing

All behavioural testing was conducted on PND 30, 60 and 120 (+/-) 5 days. Three behavioural tests were used. All subjects experienced the three tests namely, an open field, y-maze and zero maze test. For all subjects, the tests were completed over a period of five days and one test a day alternated with a “resting” day. Testing took place during the light phase of their light/dark cycle. All tests were conducted in one of two experimental rooms with the same room temperature (22 ± 2 degrees C) and dim overhead fluorescent lighting that remained unaltered across trials. Each animal was eventually tested in each apparatus three times in total. The testing apparatus was thoroughly cleaned between subjects after each test using a 20% Powerquat blue cleaning solution to minimise confounding odour cues that might have been left behind by previous subjects. Male rats were tested prior to female rats in each testing apparatus at each testing stage. These last two steps were taken to ensure that the male rats were not influenced by the odour cues left by females, which is possible even after a thorough cleaning

2.4.3 Open Field Test

The open field test was used to measure general motor and anxiety-related behaviour (Prut & Belzung, 2003). At the start, each rat was placed in the centre of the chamber and recording began about 5 seconds later. A behavioural sampling procedure was then implemented, whereby, the rat’s location (grid square that was occupied by the majority of the rats’s body) and behaviour (Walking, Grooming, Immobile, Rearing) was noted every 3 seconds for 5 minutes.

All open-field test observations were carried out by means of a small CCTV camera which was suspended over the chamber, thus allowing the experimenter to observe subjects via a television monitor distanced away from the apparatus within the same room. This was done to reduce any effect that the experimenter's presence might have on the subject's behaviour. Ambulation (a measure of activity) was measured based on the number of transitions the rat made from one square to another between the 3-second observations. This was counted and recorded at the end of the 5 minute trial.

The level of anxiety or perceived stress levels are indicated by the frequency of walking, rearing and centre occupancy (occupancy of the four centre squares). For instance, higher frequencies of walking, rearing and centre occupancy indicate lower levels of anxiety, whereas, higher frequencies of a immobility, grooming and corner occupancy (occupancy of the four corners) indicate high anxiety (Díaz-Morán et al., 2014; Prut & Belzung, 2003). Finally, at the end of the 5 minutes session, the rat was removed and returned to it's cage and the number of faecal boluses left in the apparatus was counted. This was used as a measure of emotionality, as it has been suggested that in a mildly stressful situation, emotional subject are likely to defecate as a result of emotion-induced parasympathetic activity (Sanberg et al., 2001).

2.4.4 Y maze test

The Y-maze test was used to measure short-term spatial memory (Hughes & Maginnity, 2007). At the beginning of each trial one arm of the maze contained a black insert and the other, a white insert. The location of the white insert was determined randomly for each subject within each group to ensure that each subject experienced the white insert at both the left and right sides for each of the three trial stages (PND 30, 60 and 120). Each rat was placed in the stem of the maze which was then covered by the clear Perspex lid, and it was allowed to explore freely for 5

minutes as part of the acquisition trial. The rat was then removed and placed into a separate empty cage and the maze (and inserts) were then cleaned. Both inserts were then replaced with clean black ones in order to ensure that the behaviour of the subject was not influenced by any odour cues left behind during the acquisition trial. The rat was then replaced in the stem of the maze for a retention trial, and its behaviour was observed and recorded. Each response (number of entries into and number of observations in each arm) was recorded every three seconds for three minutes. Based on these observations, the total time spent in the novel (changed) arm, total time spent in the unchanged arm, first entry into an arm (novel or unchanged), percentage of time spent in the novel arm and percentage of time spent in the unchanged arm, were able to be calculated. After three minutes, the rat was returned to its home cage and the maze was thoroughly cleaned.

It has been proposed that the occipitoparietal and occipitotemporal pathways are implicated in spatial localisation and visual object identification respectively in humans and similar pathways have also been implicated for spatial localisation and visual object identification for the working memory of rats (Dellu, Fauchey, Le Moal, & Simon, 1997). The Y-maze is used to measure the spatial memory of rats by exploiting their innate tendency to explore novelty (Cognato et al., 2010; Dellu et al., 1997). Rats generally enter the novel (changed) arm of the maze more frequently than the unchanged arm, however, anxious rats tend to spend more time in the unchanged, familiar arm (Hughes & Maginnity, 2007).

2.4.5 Zero Maze Test

At the beginning of the trial each rat was placed in one of the closed areas and were immediately timed to record the latency period of emergence into either open area. The recording of observations began as soon as the subject emerged into an open area (all four paws

in the open). All subjects were given up to 5 minutes to emerge, if any failed to emerge within this time, the trial was terminated, the subject was declared “D.E” (did not emerge) and returned to its home cage. After the initial emergence into an open area, the number of entries into and observations in open and closed areas were recorded every three seconds for five minutes. These measures were then used to calculate the percentage of entries into, as well as time spent in the open arms for each subject. Finally, the subject was returned to its home cage and the maze was thoroughly cleaned before the next subject was tested.

The principle behind the zero maze task is akin to that of the y-maze task in that both demand unconditioned, approach-avoidance tasks that rely on the rat’s innate aversion to open spaces coupled with their tendency to explore novelty (Tucker & McCabe, 2017). In the zero maze, the darker, closed areas are considered safe by rats, whereas, the bright and exposed open areas are considered potentially dangerous and thus anxiety-evoking (Tucker & McCabe, 2017).

3.0 Results

3.1 Maternal Weight, Fluid Intake and Methadone Dose

During lactation, maternal weights were gathered weekly in order to determine the dose consumed and assess maternal weight gain. However, only data for the first week was used to make the necessary calculations as the data for the subsequent weeks may have been contaminated by the possibility that the infant rats drank some of the water directly from the bottle. Methadone exposed dams had a mean weight of 228.17g (SEM \pm 4.76g) and control dams had a mean weight of 231.08g (SEM \pm 5.60g). A t-test for independent samples revealed that the difference in maternal weight gain between the methadone-exposed and unadulterated water-exposed dams were not significant, $t(9) = 0.40$, $p < 0.6$.

The fluid intakes of all rat mothers during lactation were recorded and the mean dose of ingested methadone calculated. The daily dose turned out to be higher than initially planned, namely, the mother rats received 4.86 (SEM \pm 0.53mg) mg/kg/day. During this first week, no significant differences were found in the amount of fluid consumed between dams drinking unadulterated water (20.14 ± 1.23 ml/100g/day) and methadone dosed water (16.21 ± 1.78 ml/100g/day), $t(9) = 1.74$, $p > 0.1$.

3.2 Behavioural and Cognitive Measures

3 separate three-way ANOVAs were conducted at PND 30, 60 and 120 for each measure (Open field, Y-maze and Zero maze) and post hoc analyses were conducted using the Scheffe post hoc test. Given the exploratory nature of the experiment, the use of these tests is considered reasonable (Cohen, 2008). All analyses were performed using the StatView package for macintosh computers.

3.2.1 Open Field Results

Table 1. Mean (\pm SEM) values of all open-field measures for control and drug-treated, non-handled and handled, and male and female rats, and results of ANOVAs.

Measure + PND	Control	Drug	F(1,56)	Non-handled	Handled	F(1,56)	Male	Females	F(1,56)
Ambulation									
PND30 ^a	44.96 (2.46)	49.97 (1.48)	2.52	42.32 (2.28)	52.03 (1.35)	17.43***	44.75 (2.05)	50.14 (1.80)	3.29
PND60 ^a	44.71 (3.10)	45.92 (2.33)	0.09	39.21 (3.31)	50.19 (1.76)	14.61***	38.82 (2.35)	50.50 (1.82)	14.28***
PND120	39.79 (2.72)	38.86 (2.05)	1.67	38.21 (2.42)	40.08 (2.26)	1.67	30.89 (2.05)	45.78 (1.84)	28.68***
Centre occupancy									
PND30 ^b	3.82 (0.51)	4.53 (0.44)	0.39	3.00 (0.39)	5.17 (0.45)	11.04**	4.50 (0.48)	4.00 (0.46)	1.77
PND60	3.79 (0.67)	5.22 (0.62)	1.28	3.57 (0.72)	5.39 (0.58)	5.23*	3.96 (0.50)	5.08 (0.54)	1.01
PND120	3.57 (0.56)	3.97 (0.66)	0.05	4.11 (0.64)	3.56 (0.61)	0.59	3.00 (0.51)	4.42 (0.66)	0.04
Corner occupancy									
PND30	63.46 (2.07)	61.25 (1.61)	0.16	66.00 (1.90)	59.28 (1.58)	9.4	63.79 (2.13)	61.00 (1.55)	0.78
PND60	57.50 (2.99)	60.11 (2.22)	1.18	61.46 (3.45)	57.03 (1.74)	2.41	61.61 (2.46)	56.92 (1.63)	14.28***
PND120	60.25 (2.48)	56.64 (2.41)	0.1	59.75 (2.54)	57.03 (2.41)	0.57	65.43 (2.79)	56.64 (2.41)	15.54***
Walking									
PND30	25.82 (1.29)	28.06 (0.89)	1.49	26.29 (1.42)	27.69 (0.78)	0.99	25.57 (1.08)	28.25 (1.03)	2.03
PND60 ^a	18.96 (1.51)	17.72 (1.50)	1.12	19.21 (1.42)	17.53 (1.18)	0.23	13.50 (0.93)	21.97 (1.49)	27.82***
PND120	18.32 (1.50)	17.22 (0.96)	2.72	17.21 (1.32)	18.08 (1.11)	0.35	13.43 (0.95)	21.03 (1.01)	32.65***
Rearing									
PND30	21.32 (1.93)	20.36 (1.16)	0.45	21.18 (1.56)	20.47 (1.03)	0.03	19.75 (1.35)	21.58 (1.18)	0.84
PND60 ^a	24.04 (2.35)	23.56 (1.58)	0.1	19.75 (2.30)	26.89 (1.42)	12.51***	22.11 (1.81)	25.06 (1.93)	1.69
PND120 ^c	29.25 (1.44)	27.36 (1.68)	0.45	25.82 (2.08)	30.03 (1.52)	2.59	27.79 (1.54)	28.50 (1.93)	0.12
Grooming									
PND30 ^b	5.29 (0.63)	4.61 (0.40)	0.94	5.18 (0.51)	4.69 (0.50)	0.16	5.25 (0.58)	4.64 (0.45)	0.77
PND60	1.79 (0.45)	2.42 (0.93)	1.29	2.43 (0.49)	1.92 (0.36)	1.59	2.00 (0.52)	2.25 (0.34)	0.01
PND120	1.18 (0.36)	1.56 (0.39)	0.44	1.50 (0.48)	1.31 (0.31)	0.44	1.46 (0.44)	1.33 (0.34)	0.58
Immobility									
PND30 ^a	36.54 (3.00)	39.06 (1.92)	0.07	39.86 (5.30)	34.47 (1.49)	3.81	37.75 (2.86)	36.11 (2.04)	0.09
PND60 ^a	54.82 (3.52)	54.22 (2.72)	0.03	58.25 (3.93)	51.56 (2.23)	4.58*	61.46 (2.48)	49.06(3.04)	10.72**
PND120 ^b	50.82 (2.80)	52.86 (1.90)	1.08	55.36 (2.24)	49.33 (2.21)	3.47	56.54 (2.00)	48.42 (2.27)	8.19**
Faecal Boli									
PND30	0.18 (0.15)	0.28 (0.13)	0.13	0.04 (0.04)	0.39 (0.17)	2.67	0.32 (0.18)	0.17 (0.10)	1.14
PND60	2.07 (0.54)	1.47 (0.36)	0.1	1.86 (0.49)	1.64 (0.41)	0.18	3.36 (0.52)	0.47 (0.21)	33.41***
PND120 ^{ad}	1.57 (0.40)	1.33 (0.32)	1.54	1.32 (0.36)	1.53 (0.35)	0.53	2.57 (0.40)	0.56 (0.23)	20.99***

*p < 0.05; **p < 0.01; ***p < 0.001.

^adrug x handling interaction significant (see text); ^bdrug x sex interaction significant (see text); ^chandling x sex interaction (see text); ^ddrug x handling x sex interaction (see text).

3.2.1.1 Main effects

As can be seen in Table 1, several main effects were found for Handling and Sex but not for Treatment effects. Subjects that were handled displayed a significant increase in the rate of ambulation and central occupancy at both PND 30 and 60. Similarly, rearing at PND 30 was significantly higher in handled subjects than non-handled subjects. Main effects were found for corner occupancy and immobility. As shown in Table 1, handled subjects displayed significantly

lower levels of corner occupancy and immobility than non-handled subjects. Furthermore, main effects for sex were found. At both PND 60 and PND 120 females had displayed significantly higher rates on ambulation and walking than males, whereas, males at PND 60 and 120 had spent significantly more time in the corners, as well as displayed significantly higher rates of immobility and produced significantly more faecal boluses than females.

3.2.1.2 Ambulation

There was a significant two-way interaction between treatment and handling on the rate of ambulation $F(1, 56) = 5.92, p < 0.05$ at PND 30 and PND 60, $F(1, 56) = 6.63, p < 0.05$. As shown in Figure 1, the interaction at PND 30 indicates that, methadone treatment significantly decreased ambulation for non-handled rats, but had no effect for handled animals. Whereas at PND60, post hoc analyses show that there were significant differences between the Handled and Non-handled groups in the Control condition as well as a significant difference between the Control and Methadone conditions in the Handled group. As shown in Figure 1, animals in the Handled group had significantly higher mean rates of ambulation than those in the Non-handled group and that methadone significantly reduced the rate of ambulation of handled animals.

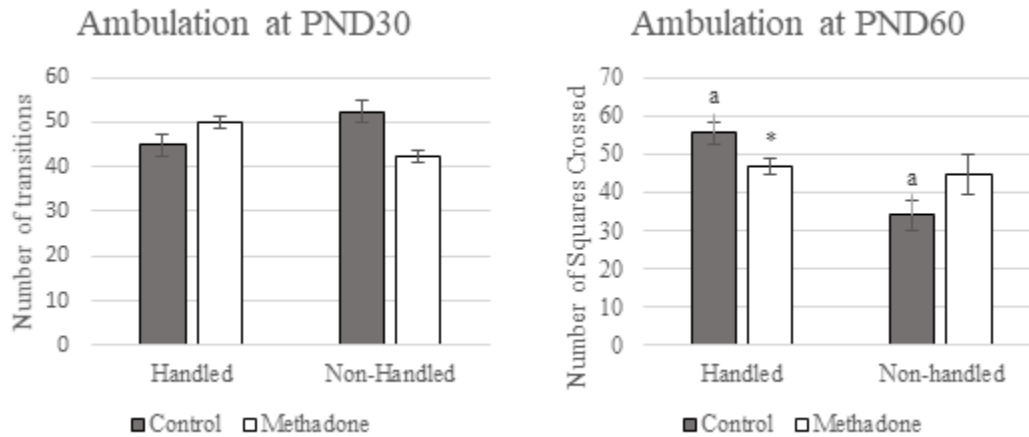


Figure 1. Mean \pm S.E.M values for ambulation at PND30 and 60 for treatment x handling interactions. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.1.3 Centre Occupancy

At PND 30 there was a statistically significant two-way interaction between treatment and sex for centre occupancy, $F(1,56) = 4.86$, $p < 0.05$. Post hoc analysis revealed that there was a significant difference in central occupancy between males and females in the control group but not in the methadone exposed group as well as a significant difference between the methadone and control groups for females. As shown in Figure 2 below, control males occupied centre squares significantly more than females in the control group, and methadone-exposed females occupied more centre squares than control females.

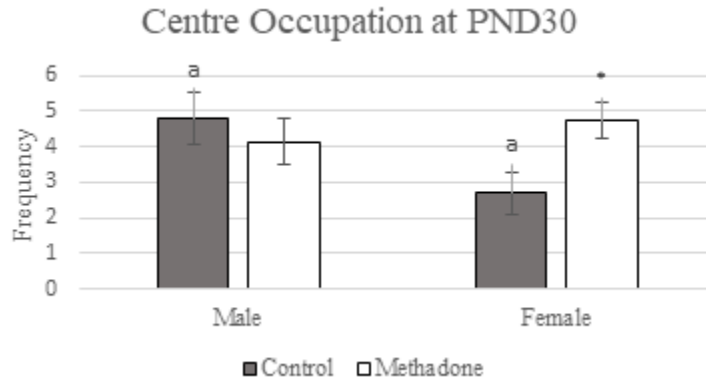


Figure 2 Mean \pm S.E.M values for centre occupancy at PND30 for treatment x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.1.4 Walking

There was a statistically significant two-way interaction effect at PND 60 between treatment and handling for walking frequency, $F(1, 56) = 8.03$, $p < 0.01$. Post hoc analyses revealed that there was a significant difference between the methadone and the control group in the Handled condition but not the non-handled condition. Figure 3 below illustrates that rat offspring in the Control group had a higher walking frequency than the Methadone group within the handled condition.

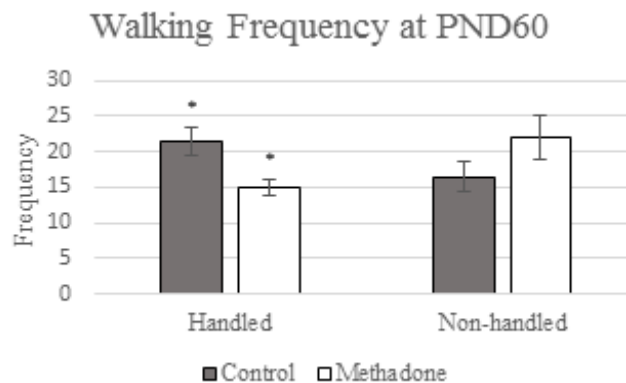


Figure 3 Mean \pm S.E.M values for walking frequency at PND60 for treatment x handling interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.1.5 Rearing

A statistically significant two-way interaction was found at PND 60 between treatment and handling for the number of rearing behaviours, $F(1, 56) = 10.93, p < 0.01$. Post Hoc analysis showed that there was a significant difference between the control and methadone groups in the handled condition but not the non-handled condition. Figure 4 illustrates that the average number of rearing behaviours was significantly lower in the non-handled condition than the handled condition for both control and methadone groups. At PND 120, a statistically significant two-way interaction was found between Handling and Sex for rearing behaviours, $F(1, 56) = 4.88, p = 0.03$. Post Hoc analysis revealed that there was a significant difference between the handled and the non-handled conditions for females but not for males. Figure 4 illustrates that females in the handled condition exhibited more rearing than females in the non-handled condition.

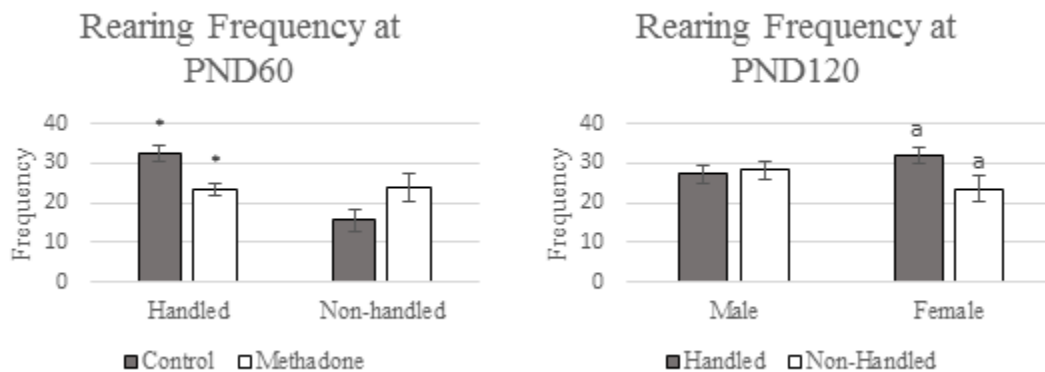


Figure 4 Mean \pm S.E.M values for rearing frequency at PND60 for treatment and handling and PND120 for handling x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.1.6 Grooming

There was a statistically significant two-way interaction at PND 30 between treatment and sex for Grooming, $F(1, 56) = 6.59, p = 0.013$. Post hoc analysis revealed that there was a significant sex differences in the control condition as well as a significant difference between

control and methadone condition within the male group. As shown in Figure 5, on average, male rats in the control condition exhibited more grooming behaviours than male rats in the methadone condition.

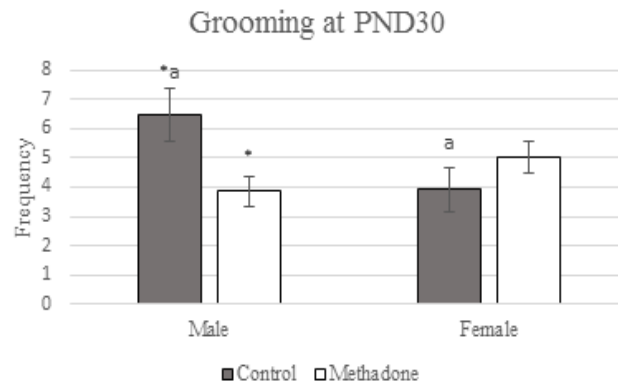


Figure 5 Mean \pm S.E.M values for grooming frequency at PND30 for treatment x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.1.7 Immobility

Significant two-way interaction was found between treatment and handling on immobility scores at PND 30, $F(1, 56) = 5.22$, $p = 0.02$ and PND 60, $F(1, 56) = 11.53$, $p = 0.013$. Post hoc analysis for the interaction at PND 30 showed that there was a significant difference between the average number of times the rats in the handled group and non-handled group remained immobile in the control condition, but this difference was not significant in the methadone condition. Figure 6 below shows that on average, rats in the non-handled, control condition, remained immobile significantly more times than the handled rats in the control condition. Additionally, at PND 60, the post hoc analysis revealed a significant difference for immobility between the control and methadone groups in the handled condition, but not the non-handled condition. As illustrated in Figure 6, non-handled, methadone-exposed rats remained immobile significantly more than non-handled control rats. At PND 120, a statistically significant two-way interaction was found between treatment and sex on immobility scores, $F(1,$

56) = 5.30, $p = 0.025$. Post hoc analysis showed that there was a significant difference between the control and methadone groups for females but not males, as well as a significant difference between males and females in the control condition. Figure 6 illustrates that on average the females in the methadone group has significantly higher immobility scores than the females in the control group. The figure further illustrates that males in the control condition had significantly higher immobility scores on average than the females in the control condition.

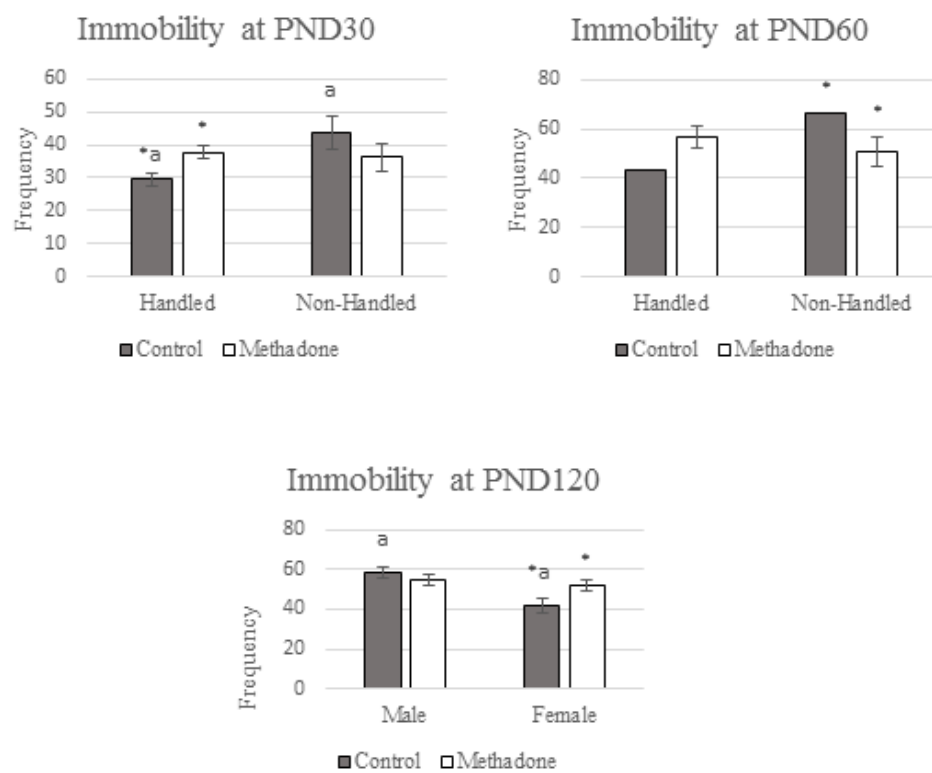


Figure 6 Mean \pm S.E.M values of immobility frequency at PND30 and PND60 for treatment x handling interactions and at PND120 for treatment x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.1.8 Faecal Boluses

At PND 120, there was a statistically significant three-way interaction between treatment, handling and sex on the number of faecal boluses, $F(1, 56) = 5.07, p = 0.028$. Post hoc analysis revealed that there is a significant difference in the average number of faecal boluses produced by the non-handled male rats between the control and methadone conditions. Figure 7 illustrates that male rats in the non-handled control condition produced significantly more faecal boluses than non-handled males in the methadone condition.

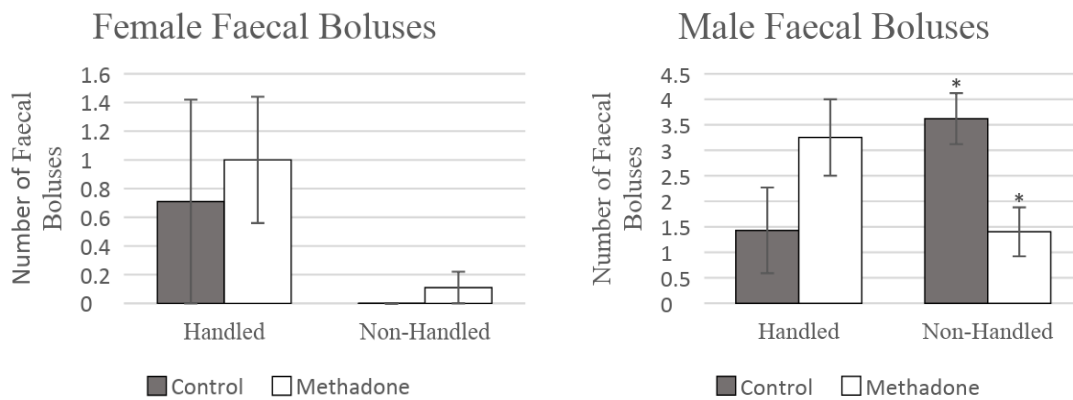


Figure 7 Mean \pm S.E.M values for the number of faecal boluses at PND120 for treatment x handling x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.2 Y-Maze Findings

Table 2. Mean (\pm SEM) values of all Y-maze measures for control and drug-treated, non-handled and handled, and male and female rats, and results of ANOVAs.

Measure + PND	Control	Drug	F(1,56)	Non-handled	Handled	F(1,56)	Male	Females	F(1,56)
% Occupancy of Novel Arm									
PND30 ^a	62.43 (3.48)	55.82 (3.29)	4.19*	55.17 (4.84)	61.46 (2.02)	2.16	55.82 (4.19)	60.95 (2.79)	2.51
PND60	56.21 (2.68)	59.84 (1.92)	1.11	57.81 (2.59)	58.59 (2.04)	1.72	58.84 (2.58)	57.79 (1.81)	0.37
PND120	61.74 (2.38)	64.35 (2.57)	0.73	63.43 (2.95)	63.03 (2.21)	0.01	64.56 (3.20)	62.15 (1.98)	0.3
% Entries of Novel Arm									
PND30 ^{bc}	57.26 (2.21)	58.41 (2.71)	0.2	57.48 (3.69)	58.24 (1.47)	0.45	53.61 (3.04)	61.24 (2.01)	6.23*
PND60	56.75 (1.85)	58.87 (1.41)	1.03	58.89 (1.87)	57.20 (1.41)	0.58	58.45 (1.74)	57.55 (1.51)	0.25
PND120	55.14 (1.99)	63.17 (2.17)	6.62*	57.39 (2.47)	61.41 (2.01)	1.27	60.22 (2.74)	59.21 (1.84)	0.28
Total Entries of Both Arms									
PND30 ^c	6.71 (0.54)	6.39 (0.39)	0.73	5.25 (0.50)	7.53 (0.34)	17.40***	6.75 (0.58)	6.36 (0.35)	0.23
PND60	8.21 (0.48)	8.47 (0.39)	4.9	7.93 (0.52)	8.69 (0.34)	1.02	8.04 (0.53)	8.61 (0.34)	1.23
PND120	7.68 (0.44)	6.33 (0.36)	8.14**	7.25 (0.49)	6.67 (0.65)	0.64	6.36 (0.51)	7.36 (0.31)	5.63*

*p < 0.05; **p < 0.01; ***p < 0.001.

^a drug x handling interaction significant (see text); ^b drug x sex interaction significant (see text); ^c handling x sex interaction (see text); ^d drug x handling x sex interaction (see text).

3.2.2.1 Main Effects

Main effects were found for treatment, handling and sex at different stages. As can be seen in Table 2, at PND 30, subjects in the control group displayed a significantly higher percentage of novel arm occupation than the methadone-exposed group. Additionally, at PND 120, main effects were found for both percentage of entries into a novel arm and the total entries into both arms. Rats in the methadone group made a significantly higher number of entries into the novel arm than the control rats, whereas, control rats made significantly higher number of entries into both arms. Table 2 further shows that handled rats at PND 30 had a significantly higher amount of total entries into both arms than the non-handled rats. Main effects for sex were also found. At PND 30, females had a significantly higher percentage of entries into the novel arm than males and at PND 120, females had a significantly higher number of total entries into both arms.

3.2.2.2 % Occupancy of Novel Arm

A statistically significant two-way interaction was found at PND 30 between treatment and handling on the occupation of novel arm, $F(1, 56) = 4.27, p = 0.034$. Post hoc analysis revealed that there was a significant difference in the average occupancy scores between the control and methadone groups in the non-handled condition as well as a significant difference between handled and non-handled groups in the methadone condition. Figure 8 shows that non-handled rats in the control condition had a higher percentage of occupancy of a novel arm compared to the non-handled rats in the methadone condition. It further illustrates that handled rats in the methadone condition had a significantly higher percentage occupancy of novel arm than the non-handled rats in the methadone condition.

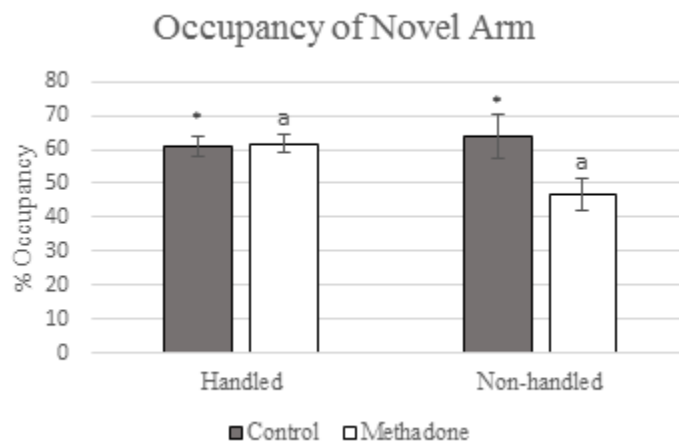


Figure 8 Mean \pm S.E.M values for occupancy of novel arm at PND30 for treatment x handling interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.2.3 % Entries into Novel Arm

At PND 30, there was a statistically significant two-way interaction between treatment and sex, $F(1, 56) = 5.38$, $p = 0.0241$. Post hoc analysis revealed a significant difference between male and female percentage of entries within the methadone condition. Figure 9 shows that females in the methadone group had a higher percentage of entries into novel arm than males in the methadone group. There was also a statistically significant two-way interaction between handling and sex, $F(1, 56) = 6.73$, $p = 0.012$. Post hoc analysis revealed a significant difference between males and females in the non-handled condition. Figure 9 shows that males in the non-handled condition have a significantly lower percentage of entries than females in the non-handled condition.

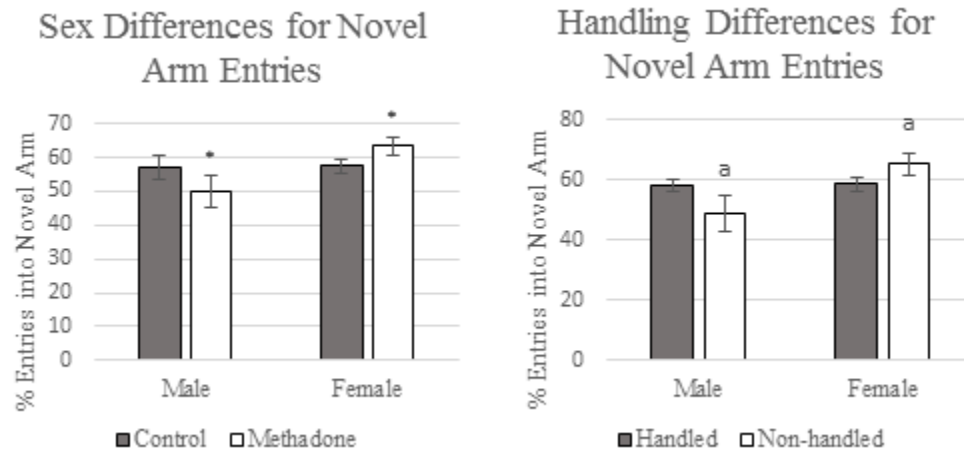


Figure 9 Mean \pm S.E.M values for percentage entries into the novel arm at PND30 for treatment x sex and treatment x handling interactions. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.2.4 Total Entries

There was a statistically significant two-way interaction between Handling and Sex at PND 30, $F(1, 56) = 6.4$, $p = 0.014$. Post hoc analysis revealed a significant difference in the average number of entries between males in the handled and non-handled groups. As shown in Figure 10, males in the handled group had a higher number of total entries than males in the non-handled group.

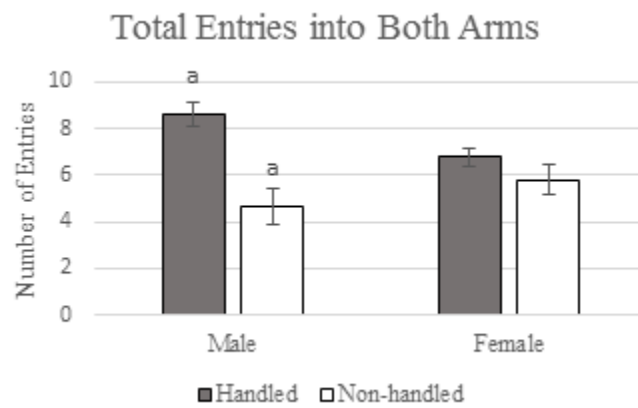


Figure 10 Mean \pm S.E.M values for total entries at PND30 for handling x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.3 Zero Maze Findings

Table 3 Mean (\pm SEM) values of all Zero maze measures for control and drug-treated, non-handled and handled, and male and female rats, and results of ANOVAs.

Measure + PND	Control	Drug	F(1,56)	Non-handle	Handled	F(1,56)	Male	Females	F(1,56)
Latency of First Entry Into Open Arm									
PND30	113.3 (14.49)	101.72 (13.53)	0.32	101.62 (13.57)	111.32 (14.26)	0.11	110.025 (12.54)	104.68 (14.3)	0.01
PND60	89.26 (12.9)	93.83 (15.32)	1.80	97.84 (15.73)	87.04 (13.51)	1.80	102.82 (15.76)	88.03 (12.60)	1.11
PND120 ^c	56.25 (11.77)	72.38 (10.20)	5.40*	78.11 (13.47)	54.90 (8.15)	5.75*	104.04 (14.96)	40.51 (4.82)	26.04***
% Entries Into Open Arms									
PND30 ^{ad}	51.01 (0.76)	53.98 (1.45)	7.93**	51.87 (1.43)	53.27 (1.11)	0.82	51.73 (1.51)	53.23 (1.08)	0.33
PND60	53.50 (2.61)	50.57 (0.66)	0.70	54.18 (2.61)	50.06 (0.51)	1.11	50.00 (0.00)	52.53 (1.63)	1.29
PND120	51.78 (0.46)	50.88 (1.05)	0.18	51.45 (0.38)	51.09 (1.16)	0.04	52.48 (1.05)	50.45 (0.79)	1.15
% Observations In Open Areas									
PND30	13.09 (2.00)	15.43 (2.79)	0.12	16.57 (3.39)	12.57 (1.60)	0.29	8.90 (1.65)	18.20 (2.57)	7.87**
PND60	22.60 (3.60)	24.48 (3.05)	0.24	28.42 (4.21)	20.15 (2.40)	0.89	14.00 (3.83)	27.56 (2.57)	6.90*
PND120 ^d	34.79 (2.40)	30.88 (2.31)	2.48	35.22 (2.96)	30.13 (2.15)	3.92	25.96 (3.02)	36.80 (1.97)	10.76**
Total Entries Of Both Arms									
PND30	7.04 (0.89)	7.24 (0.66)	0.09	6.42 (0.96)	7.79 (0.79)	3.01	5.81 (0.85)	8.06 (0.64)	5.15*
PND60	12.32 (1.60)	10.60 (1.34)	0.66	11.89 (1.49)	10.92 (1.43)	0.57	6.67 (2.02)	13.09 (1.05)	11.64**
PND120	16.08 (1.82)	13.32 (1.02)	4.47*	14.59 (1.44)	14.35 (1.33)	0.16	9.30 (1.38)	17.86 (0.97)	30.42***

*p < 0.05; **p < 0.01; *** p < 0.001.

^a drug x handling interaction significant (see text); ^b drug x sex interaction significant (see text); ^c handling x sex interaction (see text); ^d drug x handling x sex interaction (see text).

3.2.3.1 Main Effects

Several main effects were found for treatment, handling and sex for the zero maze measures. Table 3 shows that rats exposed to methadone took significantly longer to emerge into the open for the first time at PND 120, whereas, at PND 30, methadone exposed rats displayed a significantly higher percentage of entries into the open. Table 3 further shows that Methadone-exposed rats made significantly less total entries into both arms than control rats at PND 120. A main effect for handling was also found. As seen in Table 3, non-handled rats at PND 120 had

significantly higher latency periods to emerge into the open than the handled rats. There were several main effects found for sex. As Table 3 shows, the latency to emerge into the open was significantly higher for males than for females at PND 120. Additionally, at all age points (PND 30, 60 and 120), main effects were found for the percentage of time rats were observed in the open as well as the total number of entries into both arms. Female rats were found to have spent significantly more time in the open areas and had higher total number of entries into both arms.

3.2.3.2 % Entries into Open Arm

A statistically significant two-way interaction between Handling and Sex at PND 30, $F(1, 56) = 10.39, p = 0.002$. Post hoc analysis shows a significant difference between non-handled and handled groups among females but not males. Figure 11 shows that the females in the handled group made significantly more entries into the open compared to those in the non-handled group. A statistically significant three-way interaction was also found between treatment, handling and sex, $F(1, 56) = 7.23, p = 0.01$. Post hoc analysis showed that there was a significant difference between the control and methadone conditions for the males in the non-handled group but not the females. Figure 11 shows that the males exposed to methadone had higher percentage on entries into the open than the control males.

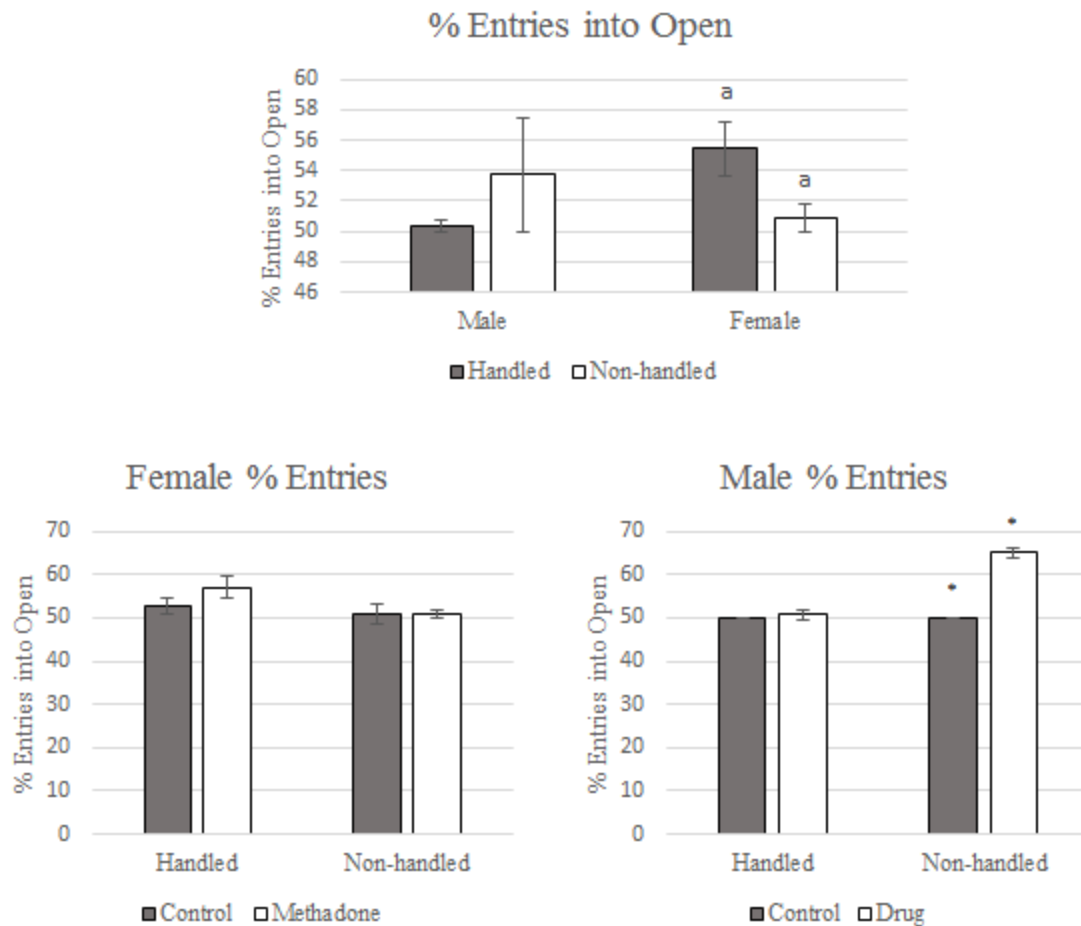


Figure 11 (above) Mean \pm S.E.M values for % entries into the open at PND30 for handling x sex interaction. **(below)** Mean \pm S.E.M values for % of entries at PND 30 for treatment x handling x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.3.3 Observed in the open areas

At PND 120, a statistically significant three-way interaction was found between treatment, sex and handling, $F(1, 56) = 13.58, p < 0.001$. Post hoc analysis revealed significant differences between control and methadone groups for non-handled females, as well as significant differences between control and methadone groups for handled males as well as differences between handled males and females in the methadone condition. Figure 12 shows

that non-handled females in the methadone group were observed in the open areas significantly less than those in the control group. Handled males in the methadone group were observed in the open areas significantly less than control handled males. Additionally, handled females in the methadone group were observed in the open areas significantly more than handled males in the methadone group.

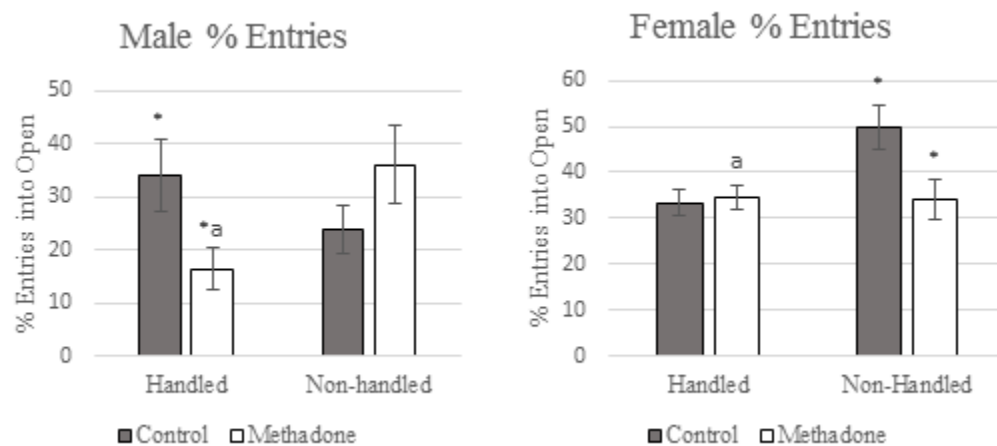


Figure 12 Mean \pm SEM values for % of entries into open by males and females at PND 120 for the treatment \times handling \times sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.2.3.4 Latency into Open

At PND 120, there was a statistically significant two-way interaction between sex and handling, $F(1, 56) = 9.04$, $p = 0.004$. Post hoc analysis revealed that there was a significant difference between males and females in the non-handled group as well as between males in the non-handled and handled groups. As illustrated in Figure 13, males in the non-handled group took a longer time to emerge into the open area for the first time than females in the non-handled group. Males in the handled group took less time to enter into the open for the first time than those in the non-handled group.

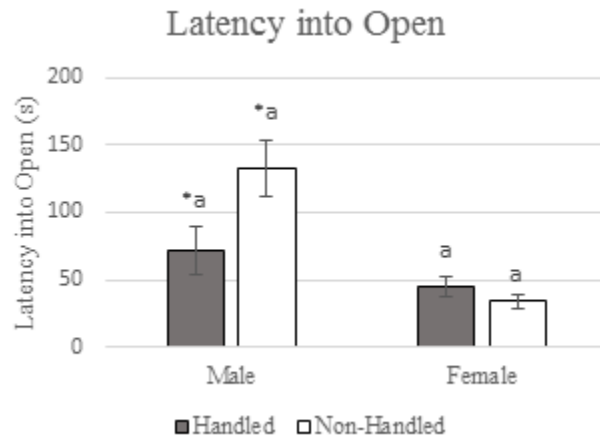


Figure 13 Mean \pm SEM values for latency into open by males and females at PND 120 for the handling x sex interaction. *significantly different ($p < 0.05$) from Control group; ^agroups with superscript in common significantly different ($p < 0.05$)

3.3 Summary Table of Interactions

Test	Age	Interaction Effects
Open Field Ambulation	PND 30	Methadone decreased the rate of ambulation for non-handled rats but had no effect on handled.
	PND 60	Handled animals had higher mean rate of ambulation than non-handled. Methadone reduced the rate of ambulation for the handled group.
Center Occupancy	PND 30	Methadone effect significant in females only. Methadone females occupied center squares more often than control females.
Walking	PND 60	Handled-methadone group had lower walking frequency than handled-control group.
Rearing	PND 60	Handled animals reared significantly more than non-handled animals in general.
	PND 120	Handled-females exhibited more rearing than non-handled females. This effect was not found in males.
Grooming	PND 30	Methadone-male rats exhibited less grooming behaviors than control-male rats.
Immobility	PND 30	Control-handled rats remained immobile significantly less than non-handled control rats.
	PND 60	Non-handled, methadone-exposed rats remained immobile significantly more than non-handled, control rats.
	PND 120	Methadone-females remained immobile more than control-females. Control-males had higher immobility scores than control-females.
Faecal Boluses	PND 120	Non-handled, control male rats produced significantly more faecal boluses than non-handled, methadone male rats.
Y-Maze % Occupancy of Novel Arm	PND 30	Non-handled, methadone rats had a lower percentage of occupancy than non-handled control rats. Handled-methadone rats had a higher percentage of occupancy than non-handled methadone rats.
% Entries in Novel Arm	PND 30	Methadone females had higher percentage of entries than methadone males. Non-handled females had a higher percentage of entries than non-handled males.
Total Entries	PND 30	Handled males had higher number of total entries than non-handled males.
Zero Maze % Entries into Open Arm	PND 30	Handled females made significantly more entries into open than non-handled females. Methadone males had higher percentage of entries than control males.
Observed in Open Areas	PND 120	Non-handled methadone females spent less time in the open areas than non-handled control females. Handled methadone males spent less time in the open than handled control males. Handled methadone females spent more time in the open than handled methadone males.
Latency into Open	PND 120	Non-handled males had longer latencies than non-handled females. Handled males had lower latencies into the open than non-handled males.

4.0 Discussion

The current study examined the teratological properties of long-term methadone exposure during lactation on anxiety and cognitive behaviours in rat offspring. However, the main focus of the study was to assess the extent to which postnatal handling can compensate for any disruption of maternal behaviour caused by methadone. The results generally supported the hypothesis that postnatal handling could have a moderating influence on the effects of methadone in terms of behaviour. This section will discuss the findings for both anxiety and spatial memory behaviour by firstly addressing the drug effects followed by the postnatal handling effects on the various testing measures as well as comment on any differences in terms of sex and age. These findings will be compared to previous research and relevant theories along with consideration of methodological limitations of the study and suggestions for future research.

Calculations during the analysis stage of this study revealed that the dosage of methadone per day was slightly higher (4.86 mg/kg/day) than the initial aim (3 mg/kg/day). However, it is unlikely that this minor difference would produce a marked effect in terms of maternal behaviour, considering other studies have used much higher doses of methadone for rat experimentation (McMillan, McGivney, Hardwick, 1980; Pierce, Hope & Raper, 1996).

4.1 Anxiety

In general, handled control rats showed a higher rate of ambulation than non-handled controls at PND 60. Handled control rats at PND30 were significantly less immobile than non-handled control rats. These findings indicate that handled rats had a lower level of anxiety than the non-handled rats, a finding that is consistent with past studies (Antoniazzi et al., 2017; Raineiki et al., 2014). Additionally, the current study found that methadone produced a

significant decrease in walking and ambulation frequency in the open field. For example, at PND 30, methadone reduced open-field ambulation for non-handled but not for handled rats. This indicates that any possible increased anxiety in methadone-exposed rats was eliminated by handling thereby suggesting that any lack of sufficient attention from the mother (rather than increased anxiety from methadone consumed in milk) may have been made up for by stimulation from handling. However, at PND 60, methadone-exposed rats in the handled condition had significantly lower activity levels than the control rats in the handled condition for both measures (ambulation and walking). This decrease in ambulation and walking frequencies suggest that methadone-exposed, handled rats may have experienced a higher level of anxiety compared to the handled controls. Taken together, these findings indicate that although additional stimulation by handling may provide some compensation for lack of maternal attention, it may only be moderately beneficial and may not be sufficient to eliminate the effects of methadone on maternal behaviour entirely.

Some drug x handling interaction effects were noted in the open field results. Methadone significantly decreased rates of walking in handled subjects at PND 60 and increased immobility in non-handled methadone subjects at PND 60. These findings indicate an increase in anxiety-like behaviour for methadone-exposed handled as well as non-handled subjects and fail to support the hypothesis of this study. In support of the point made previously, while handling seemed to have improved anxiety levels at PND 30, its effects do not appear to continue to be advantageous later in life. Moreover, as methadone had also increased anxiety-like behaviour in non-handled animals, there is cause to speculate that the behavioural outcomes may be predominantly a direct effect of the drug as opposed to impaired maternal care. Additionally, some interesting sex differences were found. For example, handled females displayed more

rearing behaviour than non-handled females at PND120. These effects seemed to have lasted until adulthood for females. However, as this was only observed in the rearing measure, it is difficult to evaluate this with respect to anxiety in general. Male controls were found to have remained immobile longer than female controls, however, control males had occupied more centre squares than control females. This finding suggests that although male rats are less active than female rats in adulthood, their levels of anxiety are lower in comparison.

The zero maze findings in this study highlighted mainly sex differences. Methadone-exposed handled females spent more time in the open areas than methadone-exposed handled males at PND 120. Females displayed lower levels of anxiety than males. In general, this finding is consistent with past studies examining activity and anxiety levels in rats. Females are usually shown to be more active and less fearful than their male counterparts (Archer, 1975; Daly et al., 2012; Gray & Hughes, 2015). Tucker and McCabe (2017) suggested that this could have been due to estrous cycle factors in female rats. Higher levels of both exogenous estradiol and progesterone have been shown to be related to lower levels of anxiety and these hormone levels are highest during proestrus and estrus (J. J. Byrnes, Babb, Scanlan, & Byrnes, 2011; Gray & Hughes, 2015). However, these sex differences in anxiety-like behaviour may be confounded by variability in locomotor activity rather than the estrus cycle of female rats, as it has been demonstrated in the past that the use of female mice at undetermined stages of the estrus cycle does not contribute to the variability in the data of behavioural experiments (Tucker and McCabe, 2017). Non-handled males at PND 120 took longer to emerge into the open than non-handled females.

Moreover, handled female rats at PND 120 spent more time in the open than handled males. These findings illustrate that male rats were reluctant to explore a novel and brightly lit

environment, whereas female rats were more willing to surrender to their natural tendency to do so. These findings lend further support to previous findings which have found that neonatal handling differentially affects anxiety-like behaviour in males and females (Weinberg, Krahm, & Levine, 1978). In addition, studies have suggested that males and females have a differential molecular response to disruptions in maternal care, yet the factors that might contribute to these differences are not clearly understood (Curley and Champagne, 2016). Nevertheless, the current study found that handled females showed higher percentages of entries into the open areas than non-handled females at PND 30 and handled males emerged into the open sooner than non-handled males. This suggests that the handled rats experienced lower levels of anxiety than non-handled rats regardless of sex, thus indicating that handling may have had a positive effect in keeping anxiety levels low. These results are consistent with the hypothesis of this study and with previous research into the effects of postnatal handling on anxiety levels (Antoniazzi et al., 2017; Li, Lund, & Voigt, 2016).

Studies have also shown that anxiety responses in the elevated zero maze and fearfulness-related responses in the open field test are strongly associated, hence, the use of both these tests in the present study was necessary in order to test the validity of a given response (Diaz-Moran et al., 2014). For example, Diaz-Moran et al (2014) found that higher levels of open field freezing (immobility) and reduced ambulation were associated with shorter zero maze time in the open and lower entries. This association is consistent with the results of the present study.

Largely, methadone-exposed animals displayed more anxiety-like behaviour than control animals and handled animals displayed fewer anxiety-like behaviour than non-handled animals. The increase in anxiety levels found in the methadone-exposed groups compared to controls in this study could be a result of the drug's direct effects on maternal behaviour, as suggested in the

introduction. In humans, several studies have reported disrupted maternal behaviour while taking opioids, in particular, increased abuse and neglect (Freisthler, Wolf, Wiegmann, & Kepple, 2017; Maguire et al., 2016). Additionally, mothers who have abused heroin are more likely to display depressed and withdrawn behaviour during interactions with their children and thus tend to be less positive towards them (Solis et al., 2012). Opioids have been found to alter maternal behaviour in rat dams treated with morphine sulphate, whereby the dams took significantly longer to engage in nurturing behaviours such as nursing and grooming (Yim et al., 2006). Studies have found that nursing, contact and tactile stimulation from the mother as a result of neonatal handling could function to suppress HPA action in the neonate by maintaining the low levels of glucocorticoids during the stress hyporesponsive period (SHRP) that neonates experience following birth (Rainekei et al., 2014; Heiderstadt, McLaughlin, Wright, Walker, & Gomez-Sanchez, 2000). Additionally, studies have found that rats that have received regular stimulation showed a reduction in emotional behaviour and that the emotional wellbeing of the mother strongly influences the stress reactivity of the offspring (Ader, 1966; Champagne et al., 2003). The findings of the current study concur with the aforementioned research and support the hypothesis that postnatal handling has the potential to ameliorate disruptions in maternal behaviour as a consequence of methadone treatment in terms of anxiety-like behaviour in rat offspring.

4.2 Spatial memory

The ability to recognize the novel arm in a Y-maze depends on spatial memory for locating which of the two arms has changed between trials (Hughes and Maginnity, 2007). The animal needs to make associations among the spatial environmental cues to form a cognitive map that helps them find the previously unvisited arm (Soares et al., 2013). Increased entries and time

spent in a novel arm indicate that the rat recognized the novel arm as the unvisited arm in the previous trial. It is important to note that no significant results were found beyond the adolescent stage at PND 30, which indicates that the effects of methadone via lactation are unlikely to have long-term spatial memory effects.

Several significant effects were noted for the Y-maze testing paradigm. For example, methadone-exposed handled rats had a higher percentage of occupation of the novel arm than non-handled methadone rats. Handled males had a higher number of total entries into both arms than non-handled males. These findings indicate that handled rats seem to have experienced less spatial memory deficits than non-handled rats as the increase in time spent in the novel arm indicates they recognize it as the changed arm. This could suggest that additional stimulation by handling may eliminate any potential spatial memory deficits which could be attributed to inadequate maternal care. Postnatal handling therefore appeared to enhance the spatial memory of methadone-exposed rats, a result that is consistent with past animal and human research (Rainecki et al., 2014; Antoniazzi et al., 2017; Konijnenberg et al., 2016; Bernstein and Hans, 1994). One explanation for this result could be that postnatal handling increases BDNF levels in the hippocampus which is associated with improved spatial memory in males (Garoflos, Stamatakis, Mantelas, Philippidis, & Stylianopoulou, 2005).

Some sex differences were also found. Methadone-exposed females made more entries of the novel arm than methadone-exposed males in both handled and non-handled groups, indicating that males may be more susceptible to spatial memory deficits than females when exposed to methadone postnatally rather than due to a lack of maternal care. This conclusion is consistent with previous research into pre- and postnatal opioid exposure (Chen et al., 2015; Fodor et al., 2014; Timár et al., 2010). Certain brain areas such as the hippocampus and the

posterior parietal cortex have been identified as structures that are crucial to spatial memory (Stuchlik, Kubik, Vlcek, & Vales, 2014). Previous studies have found that morphine exposure during the early life of newborn male rats can modulate brain-derived neurotrophic factor (BDNF) expression in the hippocampus which may be associated with behavioural alterations (Rozisky et al., 2013). It is possible that the differences between male and female results in the present study could be a result of these alterations. However, the effect of opioids specifically on female BDNF expression in the hippocampus has yet to be confirmed. Therefore, whether or not the difference between males and females based on this reasoning exists is yet to be determined. However, there are contradictory findings for the effects of postnatal handling on female hippocampal BDNF levels (Reis et al., 2014). Additionally, non-handled males had a lower percentage of entries into novel arm than non-handled females. The ability of rats to detect and explore the changed Y-maze arm has been shown to involve the influence of egocentric and allocentric cues on spatial memory (Hughes and Maginnity, 2007). Male rats are more likely to use allocentric, visual cues, whereas females are more likely to use egocentric cues. However, whether or not postnatal handling could have exerted any effects on these tendencies is unclear.

4.3 Limitations and Future Directions

While there were a number of significant findings in the present study, there were a number of limitations that may have compromised some of the results and thus affected the accuracy of some of the conclusions made. Firstly, the daily dose of methadone consumed by the rat dams was not consistent. Rat dams were weighed weekly instead of daily, which may have compromised the accuracy of the estimation of the daily dose of methadone. Having a more accurate estimation of the dose would have strengthened the ability to attribute methadone treatment to maternal and offspring behavioural effects (Daly et al., 2012). Although past

research has suggested that rat dams be measure more often during the week to obtain more accurate dose information, this was not carried out in the present study as birth rates for dams at the beginning of the experiment were low. Therefore, rat dams were measured weekly in order to reduce stress levels which otherwise could have further compromised birth rates. Another factor that could have affected the dose intake and subsequently the results is that the mothers and pups were placed in cages together until the pups reached PND30 and potentially shared the drinking bottle during this period as soon as the pups were able to reach it. This means that it is possible that some if not all pups in the methadone groups could have had a regular, direct dosage of methadone themselves from the shared drinking bottle. In future research, perhaps the mothers and pups ought to be housed in the same cage separated by a wire mesh and have separate bottles once they are able to reach the bottle for themselves. However, this separation would prevent maternal behaviour and disrupt the quality of care that the mother can provide.

Secondly, rat dams in the present study were housed individually in order to accurately calculate the amount of water each dam drank as well as the dosage of methadone consumed. However, this isolation may have potentially increased stress levels within the dams as rats are generally social creatures which have evolved to live in large colonies for security. Past research has found that the quality of the maternal environment is associated with variations in maternal behaviour (Champagne et al., 2003). Chronic stress could increase anxiety and fearfulness within the dam, and subsequently, decrease maternal responsivity which could then influence the development of stress reactivity in the offspring. Perhaps future research should adopt a design which would allow dams to be housed in grouped cages in order to eliminate the effects of isolation and be given fixed doses of methadone to individual dams via the use of a syringe. The

use of a syringe-method for oral dosing rats has been found to be effective in the past (Atcha et al., 2010). This method would also allow for an accurate estimation of the dose consumed.

Thirdly, the birth rates for the current study were variable for each rat dam, the litter sizes ranged from 1 pup to 9 pups per mother. This difference in litter size could have affected the amount of maternal attention received by each pup. Studies have found that maternal behaviour can vary depending on the sex composition of their litter, which can subsequently affect anxiety levels in the offspring (Kosten, Huang, & Nielsen, 2014). Hence, the consistency of maternal behaviour such as licking and grooming as well as the amount of breast milk received by the offspring in the current study may have been variable. Additionally, it would have been more informative to observe maternal behaviour after handling. The amount of licking, grooming and nursing was not observed or recorded in the present study. Such details could have provided more insight into the effects of postnatal handling. Again, this was avoided to reduce stress levels. In future research, it may be useful to observe and record these behaviours through the use of a video camera, thus eliminating any intrusive examiner effects.

4.4 Implications and Conclusion

The current study supported several previous findings around the effects of postnatal handling on anxiety and spatial memory. Although the conclusions made are not particularly robust, the findings are nevertheless somewhat consistent and informative. It would seem that inadequate maternal behaviour as a result of methadone consumption can potentially have long-term effects on anxiety and spatial memory; however, any effects that methadone might have had on spatial memory did not persist beyond the adolescent stage of life. Postnatal handling effects were observed to have moderated the effects of methadone up until young adulthood for anxiety and until adolescence for spatial memory. Additionally, significant drug x sex interactions were

prominent in the current study. Males seemed to have experienced higher levels of anxiety and more spatial memory deficits than females. These findings provide further support for the importance of including both male and female subjects within animal research.

In terms of the implications that these findings might have for humans, the results suggest that methadone exposure through breast milk may result in negative behavioural consequences in and of itself. Furthermore, based on the drug x sex interactions found, methadone exposure may have differential effects on female and male children which may possibly continue into adulthood. Such differences might include hyperactivity in girls (increased locomotion of female rats) and depression and anxiety in boys (immobility and reluctance to spend time in the open areas in male rats). Overall, these results suggest that the quality of maternal behaviour received can potentially influence and moderate the negative effects of methadone and could inform the best practice of methadone maintenance treatments for mothers addicted to opioids. While methadone exposure via breast milk may be a factor for potentially harmful behavioural outcomes, this study has highlighted that positive maternal behaviour has the potential to minimise such outcomes. Accordingly, perhaps OMT treatment programs could include an educational element to inform methadone-treated women about the benefits of positive maternal behaviours.

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